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## Insights into Antifolate Resistance from Malarial DHFR–TS Structures

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*Plasmodium falciparum dihydrofolate reductase-thymidylate synthase (PfDHFR–TS) is the main target of antimalarial antifolate drugs like pyrimethamine or cycloguanil, which inhibit the DHFR part of the bifunctional enzyme. Resistance to this class of drugs is now widespread due to mutations which result in lower inhibitor binding affinities of DHFR. Scientists from the BIOTEC Center and Mahidol University in Thailand, and the University of Edinburgh, UK, have obtained the first crystallographic structures of wild-type and mutant PfDHFR-TS in complex with either pyrimethamine or WR99210, an inhibitor with high binding affinities with both wild-type and mutant enzymes. This research reveals the binding modes of the inhibitors with the target, and gives insights into the basis of antifolate resistance and possible approaches to the design of new drugs to overcome the resistance.*

Antifolate antimalarials such as pyrimethamine and proguanil, a prodrug of cycloguanil, have long been used clinically in the treatment of malaria infection, especially that due to *Plasmodium falciparum*. The drugs act by inhibiting the dihydrofolate reductase activity of the *P. falciparum* enzyme dihydrofolate reductase–thymidylate synthase (PfDHFR–TS), and consequently preventing dTMP production and DNA synthesis. However, these drugs as well as their combinations with sulfa drugs have been compromised by parasite resistance. It is generally accepted that the resistance generally arises from mutations in PfDHFR–TS, first at residue 108 and subsequently at other residues, including 51, 59 and 164, resulting in increasingly poorer binding affinities of the enzyme with the inhibitors. However, WR99210, which differs from the compromised inhibitors in having a flexible side-chain, still binds tightly with the mutant enzymes and retains its antimalarial efficacy. Understanding the structural basis of interaction between drugs and the PfDHFR-TS, and the differences that determine drug efficacy, is important for the potential development of novel antimalarial drugs.

Structures of the wild-type, double mutant (C59R+S108N) and quadruple mutant (N51I+C59R+S108N+I164L) forms of PfDHFR–TS have been determined in complex with either pyrimethamine or WR99210. The enzyme is a homodimer, with two TS domains (288 residues each) interacting extensively to form two active sites similarly to TS from other species. The DHFR domain (231 residues) is attached to each TS directly and also through interaction with the junction region (89 residues). While sharing overall features with DHFR from other species, PfDHFR has two extra inserts that interact with the TS domain and the junction region. The junction region moreover interacts extensively with both the TS and DHFR domains. These interdomain interactions help to pull the two DHFR domains closer to one another than those of *Leishmania major* DHFR–TS, the only other homologous, bifunctional enzyme with known structure, and are probably responsible for the previously known fact that PfTS needs the presence of both DHFR and the junction region in order to express its activity. In addition, there are linings of positive electrostatic potentials on the molecular surface contributed by conserved

*From left to right, Jarunee Vanichtanankul, Penchit Chitnumsub, Jirundon Yuvaniyama, Yongyuth Yuthavong, Worachart Sirawaraporn, and Sumalee Kamchonwongpaisan.*

basic amino acids that trace the paths between the DHFR and TS active sites. These may function as surface electrostatic channels that lead dihydrofolate from TS to DHFR active sites similar to what observed in the *L. major* DHFR–TS structure. Such substrate channeling may serve to promote effective dTMP production in the synthesis cycle.

The structure of the double-mutant enzyme with bound pyrimethamine shows that the S108N mutation causes steric conflict for binding of the rigid *p*-chlorophenyl side-chain of the inhibitor, especially around the Cl atom, in agreement with previous prediction from modeling studies. In contrast, the structure of the quadruple-mutant enzyme with bound WR99210 shows that the flexible (2,4,5-trichlorophenoxy)propyloxy side-chain is oriented in such a way as to avoid this steric conflict. The flexible side-chain also interacts extensively with the enzyme, mainly through hydrophobic interactions. The quadruple mutant furthermore shows movement of residues 48–51 (0.5–2.2 Å) and residues 164–167 (0.3–0.5 Å), probably as the results of the N51I and I164L mutations respectively, which widens the active-site gap between the $C_\alpha$ atoms of C50 and residue 164 from 16.0 Å to 17.3 Å. These changes likely contribute to the reduction in binding affinities of rigid inhibitors like pyrimethamine, but can be accommodated by flexible inhibitors like WR99210. The binding mode of WR99210 gives insight into design of novel inhibitors that would be capable of averting the effects of mutations which reduce the binding affinities of other, more rigid, inhibitors.

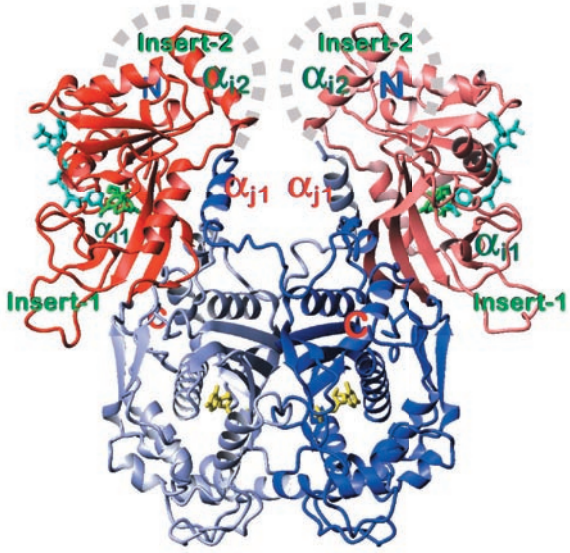


**Figure 1.** *Ribbon diagram of overall structure of the wild type PfDHFR-TS with bound WR99210, NADPH, and dUMP drawn in green, cyan, and yellow, respectively. N-terminal DHFR domains are in red while C-terminal junction regions and TS domains are in blue. N and C termini and the inserts unique to plasmodial DHFR-TS are indicated. A short helix in Insert 1 and a long helix in Insert 2 are labeled as $\alpha_{i1}$ and $\alpha_{i2}$, respectively. Termini and $\alpha_{i1}$ helix are on the back of the molecules in this orientation. The putative links between DHFR and TS domains shown as dashed gray curves are based on intermolecular spaces in crystal packing around the regions of unobserved residues. The helices $\alpha_{j1}$ in the junction region are involved in domain attachment, thus orienting the TS domains for dimerization into a functional unit.*

*Moreover, the structures provide clues to design of novel types of inhibitors which act by virtue of interference with interdomain interactions, which include interaction of TS with DHFR and Insert1, as well as electrostatic attraction between the junction-region helix and the surface groove at the DHFR–TS domain interface. Since the activity of PfTS depends on these interactions, agents which interfere with them rather than the active sites per se may also cause selective inhibition of malarial dTMP synthesis.*

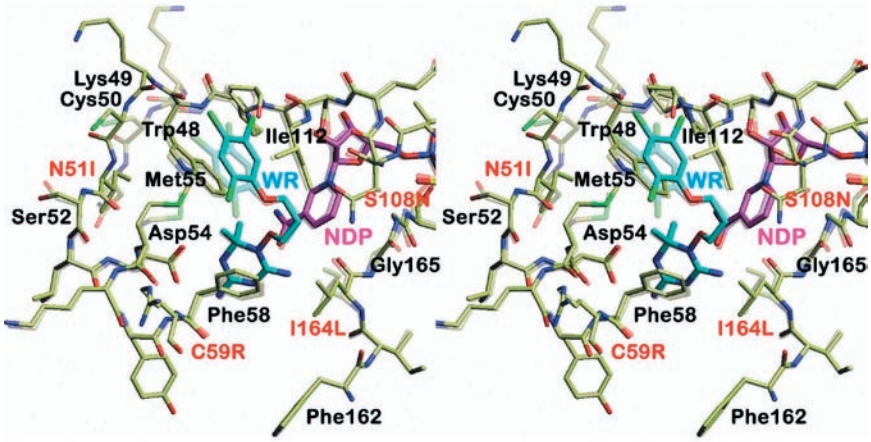


**Figure 2.** *Comparison of enzyme-inhibitor interactions at the active sites of wild-type (lighter model) and V1/S quadruple mutant PfDHFR-TS in stereo view. Both enzymes are complexed with WR99210 (WR, in cyan) and NADPH (NDP, in magenta). The flexible tail of WR99210 allows its binding in a conformation not affected by the pyrimethamine-resistant mutations (N51I+C59R+S108N+I164L) labeled in red.*